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(54) Title: PRODUCTION AND SECRETION OF RECOMBINANT FIBRINOGEN BY YEAST

(57) Abstract

The present invention relates to a novel expression system for recombinant fibrinogen, fibrinogen variants and subunits thereof, to yeasts transformed with the expression system, to the use of the expression system to clone fibrinogen, fibrinogen variants and subunits thereof, and to the use of recombinant fibrinogen, fibrinogen variants and subunits thereof as tools of research or in medicine, e.g., in diagnostic assays or as therapeutics to treat certain indications. The inventive yeast system produces at least 10 times more recombinant protein than has been possible with other expression systems. Also, the inventive yeast system is easy to adapt to produce large quantities of recombinant protein. Moreover, with the inventive yeast system, recombinant fibrinogen is the principal secretion protein and, thus, is easily purified from the culture medium.

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PRODUCTION AND SECRETION OF RECOMBINANT FIBRINOGEN BY YEAST

GOVERNMENT RIGHTS

This work supported in part by federal grant number HL37457. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a novel expression system for recombinant fibrinogen, fibrinogen variants and subunits thereof, to yeasts transformed with the expression system, to the use of the expression system to clone fibrinogen, fibrinogen variants and subunits thereof, and to the use of recombinant fibrinogen, fibrinogen variants and subunits thereof as tools of research or in medicine, e.g., in diagnostic assays or as therapeutics to treat certain indications.

2. Description of the Related Art

Fibrinogen is the soluble precursor of fibrin, which is the primary constituent of blood clots. The structure of fibrinogen has been extensively studied. For a review of the structure, see M. Furlan, in *Fibrinogen, Fibrin*

Stabilization and Fibrinolysis, J. L. Francis ed., Chichester, Ellis Horwood, 1988, pages 17-64; and R. F. Doolittle, 1984, *Annu. Rev. Biochem., 53:* 195.

Fibrinogen is composed of three different polypeptide chains (designated Aα, Bß and γ), arranged as a dimer with each half-molecule containing a set of each of the chains. The two half-molecules are linked together by three disulfide bonds at the amino-terminal portions of the polypeptides. Two of the symmetrical bonds are between adjacent γ chains and one is between Aα chains. In addition, a complex set of inter- and intrachain disulfide bonds (there are 29 disulfide bonds with no free sulfhydryl groups) are involved in maintaining proper structure.

Fibrinogen has an essentially linear shape consisting of two terminal bilobate domains tethered to a smaller central domain. The amino-termini of polypeptides α, ß and γ are contained and joined together in the central domain.

Fibrinogen is sensitive to thrombin. Thrombin cleaves peptides from the ends of the α and ß chains of fibrinogen. The released chains are referred to as the fibrinopeptides A and B, respectively. The removal of the fibrinopeptides A and B from the amino-terminus of the fibrinogen α and ß chains gives rise to an entity denoted "fibrin monomer," the spontaneous polymerization of which leads to fibrin.

Each fibrin monomer possesses specific polymerization sites (or "knobs") in the central domain that in fibrinogen are shielded by fibrinopeptides A and B. Release of fibrinopeptides A and B exposes these knobs, which are positively charged and can link with complementary negatively charged "holes" that lie on the terminal domains of neighboring molecules.

Initially, fibrin monomers polymerize to form double-stranded protofibrils, wherein the central domain of one molecule associates with a terminal domain from each of two neighboring molecules in a half-molecule staggered overlap. These protofibrils cross-link in the presence of factor XIIIa (fibrin stabilizing factor) and Ca^{2+} to form fibrin. The cross-linking is also catalyzed by thrombin, which converts the inactive enzyme precursor factor XIII to the active form factor XIIIa.

Fibrin clots are intended to be temporary sealants and, consequently, are displaced as a part of the normal wound-healing process. Plasmin degrades fibrin clots and the conversion of plasminogen to plasmin sets the pace of dissolution. The most important plasminogen conversion process involves tissue plasminogen activator (t-PA), which is released from damaged endothelial cells. t-PA on its own is not very effective in activating plasminogen, but the presence of fibrin and various fibrin breakdown products increase the activation process tremendously.

In addition to its role in clot formation, fibrinogen is also involved in platelet aggregation. High-affinity "platelet recognition sites" have been localized to the hydrophilic carboxy terminal pentadecapeptide at the opposite ends of both γ chains (residues 397-411). It appears that this segment forms a salt-bridged γ loop that fits the platelet fibrinogen receptor. After fibrinogen is bound at either γ terminal end by a platelet receptor, the molecule has sufficient "reach" to form a bridge on its free end to a fibrinogen receptor on an adjacent platelet. Subsequently, the receptors on both platelets migrate towards one another, thereby reinforcing the bridge. Other platelets are similarly engaged, thereby leading to the formation of a lattice.

Although the primary functions of fibrinogen are clot formation and platelet aggregation, fibrinogen also interacts with a wide variety of other proteins and cells. For example, when provoked or damaged, endothelial cells that line the vascular system also bind fibrinogen. The principal binding sites on fibrinogen for endothelial cells appear to be the same as those involved in the binding to platelets. See D. A. Cheresch et al., 1989, *Cell, 58:* 945. Moreover, fibrinogen is unique among the plasma proteins in being able to "clump" certain strains of *Staphylococcus aureus*. The principal "clumping" sites on fibrinogen also appear to be the same as those involved in the binding to platelets. See J. Hawiger et al., 1982, *Biochem., 21:* 1407.

The principal—and perhaps only—site of fibrinogen biosynthesis in mammals is the liver. Hepatocytes are the principal site of synthesis and each of the component chains of fibrinogen is encoded by a separate gene. These genes are expressed, the chains associate, the appropriate disulfide bonds are formed, and hexamers are released into the circulation and transported to the endoplasmic reticulum, where they are glycosylated, phosphorylated, and sulfated.

Hundreds of naturally occurring fibrinogen variants have already been identified because of their clinical consequences or during routine screening. These variants have contributed much to the understanding of fibrinogen-fibrin biochemistry, in many instances providing important insights into structure-function relationships. As valuable as naturally occurring variants have been, they will soon be overshadowed by site-directed mutagenesis experiments with recombinant fibrinogens expressed in recombinant systems.

For example, in order to study the role in calcium binding of disulfide bond γCys326-γCys339, one laboratory has already used site-directed mutagenesis to synthesize a polypeptide lacking Cys326 and Cys339 by expressing DNA encoding the modified polypeptide in *Escherichia coli*. See M. G. Bolyard et al., 1990, *Biochem. Biophys. Res. Comm., 174:* 853.

Interestingly, the same γCys326-γCys339 bond has also been implicated in clottability. R. Procyk et al., 1990, *Biochem., 29:* 1501-1507, showed that this bond is one of the bonds that are cleaved during a mild reduction of fibrinogen with a low concentration of dithiothreitol in the absence of calcium. A consequence of the limited reduction is a loss of clottability, which was later determined to result apparently from perturbation of carboxy-terminal polymerization sites on fibrinogen. This perturbation of the carboxy-terminal polymerization sites was, in turn, apparently a consequence of γCys326-γCys339 bond cleavage. See R. Procyk et al., 1992, *Biochem., 31:* 2273. Copending U.S. Patent Application Serial No. 07/946,826, the entire contents of which are incorporated herein by reference, teaches that fibrinogen reduced in this manner has substantial biochemical and immunological equivalency to fibrin and fibrin monomer and, therefore, is useful as a substitute for fibrin or fibrin monomer in assays requiring these species. Such assays include, for example, conventional assays for the quantitative determination of (i) fibrin monomers, (ii) plasmin activator inhibitor activity, (iii) tissue plasminogen activator activity, and (iv) immunoassays.

It is possible to use site-directed mutagenesis to construct a DNA sequence encoding such reduced fibrinogen or some other fibrinogen variant and then construct an expression vector containing such DNA, transform a suitable host with such expression vector and then induce the host to express the DNA.

Our laboratory and others have described systems in which transfected animal cells produce recombinant fibrinogen. See S. N. Roy et al., 1991, *J. Biol. Chem., 266:* 4758 (expression in COS-1 and Hep G2 cells); R. Hartwig et al., 1991, *J. Biol. Chem., 266:* 6578 (expression in COS-1 cells); and D. H. Farrell et al., 1991, *Biochem., 30:* 9414 (expression in baby hamster kidney (BHK) cells). These systems, however, only produce small amounts of fibrinogen and they are difficult to scale-up.

Recombinant fibrinogen will undoubtedly be useful as a research tool, but, in addition, will also be useful in medicine, for example, in the preparation of "fibrin glue" for wound healing or, in some cases, for infusion into hypofibrinogenic patients.

SUMMARY OF THE INVENTION

The principal object of the present invention was to provide a system for expressing recombinant fibrinogen, recombinant fibrinogen variants and recombinant fibrinogen subunits in relatively large amounts.

It was another object of the present invention to provide an expression system for recombinant fibrinogen, recombinant fibrinogen variants and recombinant fibrinogen subunits, which would be easy to scale-up.

It was another object of the present invention to provide for recombinant fibrinogen, recombinant fibrinogen variants and recombinant fibrinogen subunits and the use of such recombinant proteins in research and in medicine

as therapeutics and in diagnostic assays.

These and other objects were met with the present invention, which relates generally to an expression vector for yeast comprising at least one cDNA encoding a polypeptide chain of fibrinogen or a variant thereof. One embodiment includes an expression vector for yeast containing a cDNA encoding the Aα chain of fibrinogen or a variant thereof, a cDNA encoding the Bß chain of fibrinogen or a variant thereof, and a cDNA encoding the γ chain of fibrinogen or a variant thereof.

A second embodiment of the present invention relates to yeast transformed with such a vector.

A third embodiment of the present invention relates to a method of expressing fibrinogen or a variant or subunit thereof in yeast, comprising the steps of:

(a) constructing or obtaining an expression vector for yeast containing at least one cDNA encoding a polypeptide chain or fibrinogen or a variant thereof;

(b) transforming yeast with said expression vector and selecting for stable transformants;

(c) maintaining the transformants in a culture medium under conditions wherein fibrinogen or a variant or a subunit thereof is secreted into said culture medium; and

(d) recovering fibrinogen or the variant or subunit thereof from the culture medium.

A fourth embodiment of the present invention relates to recombinant fibrinogen or a variant or subunit thereof produced by the inventive process.

The inventive yeast system surprisingly produces at least 10 times more recombinant fibrinogen than has been possible with other expression systems. Also, the inventive yeast system is easy to adapt to produce large quantities of recombinant fibrinogen. Moreover, with the inventive yeast system, recombinant fibrinogen is the principal secretion protein and, thus, is easily purified from the culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a map of plasmid pYES2.

Figure 2 shows SDS-PAGE and Western blot analyses of human plasma and yeast recombinant fibrinogen.

Figure 3 shows SDS-PAGE and Western blot analyses of thrombin-induced clotting and cross-linking of human plasma and yeast recombinant fibrinogen.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for recombinant human fibrinogen or

variants or subunits thereof, which then can be used in research or in medicine as therapeutics or as a reagent for diagnostic assays. By "fibrinogen variants" or simply "variants" are meant polypeptides having essentially the amino acid sequence of human fibrinogen, but wherein one or more insertions, deletions, additions and/or substitutions intentionally have been made by conventional methods. By "fibrinogen subunits" or simply "subunits" are meant isolated polypeptides having the amino acid sequence of the individual α, ß or γ chains of human fibrinogen or a fibrinogen variant or any combination of such chains short of the complete molecule containing two of each of the chains. Various combinations of the chains are useful as research tools to study the biosynthesis and assembly of the functional protein.

cDNAs for the α, ß and γ chains of human fibrinogen have been isolated and characterized. See, respectively, M. W. Rixon et al., 1983, *Biochem., 22:* 3237; D. W. Chung et al., 1983, *Biochem., 22:* 3244; and D. W. Chung et al., 1983, *Biochem., 22:* 3250. However, due to code degeneracy, there can be considerable variation in nucleotide sequences encoding the same amino acid sequence. For the purposes of the present invention, such "degenerate variants" are also useful and are contemplated when reference is made to "cDNA encoding a polypeptide chain of fibrinogen". The term "degenerate variants", as used herein, refers to any DNA sequence, which, owing to the degeneracy of the genetic code, encodes the same amino acid sequence as another DNA sequence.

The present invention also provides expression vectors for producing useful quantities of purified fibrinogen or variants or subunits thereof in yeast. The term "yeast", as used herein, is intended to encompass any yeast strain, particularly strains of *Saccharomyces cerevisiae* or *Saccharomyces pombe*, as well as strains of other genera, for example, Pichia or Kluyveromyces, which have

also been employed as production strains for recombinant proteins.

In general, the vectors can comprise at least one cDNA encoding a polypeptide chain (either α, ß and/or γ) of fibrinogen or a variant thereof operably linked to regulatory elements derived from yeast. Following transformation of yeast cell lines with such vectors, the vectors can be induced to express the encoded polypeptide chain or, where the vector comprises all three chains, appropriately assembled fibrinogen or a variant thereof. Vectors for use in yeast are well known to those of ordinary skill in the art. Such vectors include so-called "shuttle vectors," which replicate in both *Escherichia coli* and yeast. A general description of such vectors is given in J. D. Watson et al., *Recombinant DNA*, 2nd ed., New York, W. H. Freeman and Company, 1992, pages 235-253. A more detailed description of such vectors, as well as of basic techniques of yeast genetics, including preparation of yeast media, strain storage and revival, strain growth and manipulation, mutagenesis, high-efficiency transformations, selectable markers, expression cassettes, replicators, promoters, leaders, terminators, signal sequences for secretion, etc., is given in F. M. Ausubel et al. (eds.): *"Saccharomyces cerevisiae."* *In* F. M. Ausubel et al. (eds.), *Short Protocols in Molecular Biology*, 2nd ed., New York, John Wiley & Sons, 1992, pages 13-1 to 13-49, the entire contents of which are hereby incorporated by reference.

In general, yeast can be grown in either liquid media or on the surface of (or embedded in) solid agar plates, but preference is given to liquid media. Yeast are best grown on liquid media containing dextrose (glucose), nitrogen, phosphorus, trace metals, and protein and yeast-cell-extract hydrolysates, which provide amino acids, nucleotide precursors, vitamins, and other metabolites that the cells would normally synthesize de novo. Instead of dextrose, the yeast can be grown on a variety of other carbon sources, for example, galactose, maltose,

fructose, and raffinose. In a particularly preferred embodiment, which will be described in more detail later, we have placed transcription under the control of the Gal-1 promoter element, which is induced with galactose. The media should be sterilized, for example, by autoclaving for 15 minutes at 15 lb/in^2, although such times should be increased when large amounts of media are being prepared. When cultured on such media, yeast cells divide approximately every 90 minutes.

Yeast vectors can be grouped into five general classes, based on their mode of replication in yeast: YIp (yeast integrating plasmids), YRp (yeast replicating plasmids), YCp (yeast centromeric plasmids), YEp (yeast episomal plasmids), and YLp (yeast linear plasmids). With the exception of the YLp, all are shuttle vectors. YIp plasmids contain selectable yeast genes, but lack sequences that allow autonomous replication of the plasmid in yeast. Instead, transformation of yeast occurs by integration of the YIp plasmid into the yeast genome. YRp plasmids contain sequences from the yeast genome which confer the ability to replicate autonomously. YRp plasmids have high frequencies of transformation (10^3 to 10^4 transformants/μg DNA), but transformants are sometimes unstable during mitosis and meiosis. YCp plasmids contain DNA segments from yeast centromeres and this greatly increases stability during mitosis and meiosis. YLp plasmids contain certain G-rich repeated sequences at their termini which function as telomeres and allow the plasmid to replicate as a linear molecule. However, for the purposes of the present invention, YEp plasmids are preferred. These plasmids contain sequences from a naturally occurring yeast plasmid called the "2μm circle." These 2μm sequences allow extrachromosomal replication and confer high transformation frequencies (~10^4 to 10^5 transformants/μg DNA). These plasmids are relatively stable during mitosis and meiosis and, consequently, are commonly used for high-level gene expression in

yeast.

In general, the heterologous structural sequence is assembled in appropriate reading frame with translation initiation and termination sequences, selectable markers and, preferably, a leader sequence capable of directing secretion of translated protein into the extracellular medium. The selectable markers in common use are wild-type genes such as URA3, LEU2, HIS3 and TRP1 and, preferably, use is made of all of them. These genes complement a particular metabolic defect (nutritional auxotrophy) in the yeast host and, consequently, successful transformants can be identified by their growth on selective media. In the preferred embodiment, which is described in more detail below, use is made of the MFα1 leader sequence, although other sequences will be similarly useful. When the MFα1 leader sequence is employed, the heterologous protein is cleaved off by the yeast KEX protein. It may be helpful, in these cases, as suggested by W. Fiers et al., "Secretion and Surface Expression in Microorganisms of Heterologous Proteins Important for Medical Research and Clinical Applications", in *Harnessing Biotechnology for the 21st Century*, M. R. Ladisch et al. eds., American Chemical Society, 1992, pages 23-25, to place between the pro-sequence and the heterologous gene sequence one or two Glu-Ala dipeptides, which facilitate the cleavage of the pro-sequence.

All of the vectors described above carry strong promoters utilized by RNA polymerase II. The promoters can be either inducible (e.g., Gal-1, Gal-10, PH05) or constitutive (e.g., ADHI, PGK or GPD). Transcription from these promoters depends on activator proteins bound to sites upstream of the transcription start site (in yeast, termed upstream activation sites or UAS). Preference is given to the Gal promoters, particularly the Gal-1 (galactokinase) promoter. The Gal promoters are regulated by the activator Gal-4, which binds to UAS upstream of

the transcription start, and the negative regulator Gal-80, which suppresses activation by the activator. Transcription from the Gal-1 promoter, for example, is massively induced when cells are grown in a medium that contains galactose as the sole source of carbon; under these conditions Gal-80 dissociates from Gal-4 and Gal-4 is bound to the Gal-1 UAS. Preference is, therefore, given to the use of the Gal-1 promoter in conjunction with media containing galactose as the sole carbon source.

Suitable yeast transformation protocols are well known to those skilled in the art; a yeast transformation kit may be purchased from BIO 101, Inc. (La Jolla, CA) and the kit protocol was followed with slight modifications in the examples given below. Derepression of the Gal-1 promoter occurs upon exhaustion of medium galactose. Crude yeast supernatants are then harvested by filtration and held at about 4°C prior to further purification.

In a preferred embodiment, we have achieved superior yields utilizing the expression vector pYES2, the details of the construction of which are described in the examples below. Vector pYES2 was constructed with all three fibrinogen cDNAs in tandem, each under the control of the Gal-1-promoter element fused with the MFα1 prepro secretion signal cascade. As will be discussed in greater detail below, recombinant fibrinogen secreted from yeast is similar to plasma fibrinogen when analyzed on polyacrylamide gels and, moreover, like naturally occurring plasma fibrinogen, recombinant fibrinogen secreted from yeast is capable of forming a thrombin-induced clot.

In the yeast system, fibrinogen is the principal secretion protein in the culture medium and, thus, is easily purified by conventional purifying methods for proteins, for example, by combinations of salting out, ultrafiltration, dialysis,

ion exchange chromatography, gel filtration, electrophoresis, affinity chromatography, etc.

It is also possible to achieve appropriately assembled fibrinogen by co-transfecting the yeast cells with each fibrinogen cDNA in a separate expression vector, rather than in tandem in a single vector. If use is made of this embodiment, then each vector should contain a different selection vector in order to facilitate selection of stable transformants containing all three cDNAs.

For the preparation of fibrinogen variants or variant subunits, advantage is taken of conventional mutagenesis techniques to alter the DNA sequence of the native cDNA(s). For example, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered "gene" having particular codons altered according to the substitution, deletion or insertion required. Where the fibrinogen variant is intended for research, cassette mutagenesis with degenerate oligonucleotides can be used to create a large collection of random mutations in a single experiment. Details of these techniques are well known to those of ordinary skill in the art and are not repeated here. However, references are made to J. D. Watson et al., *supra*, pages 191-211; F. M. Ausubel et al., *supra*, pages 8-1 to 8-25; and J. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Plainview, New York, Cold Spring Harbor Laboratory Press, 1989, pages 15-1 to 15-113, the entire contents of each of which are hereby incorporated by reference.

Thus, as mentioned previously, the γCys326-γCys339 bond has been implicated in clottability. For a variety of diagnostic assays, it is desirable to use a reagent that will not clot during the assay, but which is, nevertheless, biochemically and immunologically indistinguishable from fibrin or fibrin

monomer. According to U.S. Patent Application Serial No. 07/946,826, *supra*, a reagent suitable for this purpose is achieved by subjecting fibrinogen to a limited reduction, which, according to R. Procyk et al. 1992, *supra*, results in the cleavage of certain Cys-Cys bonds. It may be possible to prepare such "reduced fibrinogen" by recombinant methods. For example, it is possible, using oligonucleotide-directed site-specific mutagenesis procedures to modify the cDNAs encoding the various chains by replacing the codons for cysteines at the positions that are broken during the limited reduction with codons for other amino acids, for example, glycine or, perhaps, methionine, which, like cysteine is hydrophobic and contains sulfur in the side-chain.

In addition, recombinant fibrinogen and variants thereof will have use in medicine as therapeutic agents. Fibrinogen is one of the "acute phase proteins," the biosynthesis of which is markedly increased in times of trauma and insult. See, R. F. Doolittle: "The Molecular Biology of Fibrin". *In* G. Stamatoyannopoulos et al. (eds.), *The Molecular Basis of Blood Diseases*, 2nd ed., Philadelphia, W. B. Saunders Company, 1994, page 712. Fibrinogen replacement may be required in patients with liver failure or disseminated intravascular coagulation (DIC) or in patients with congenital fibrinogen deficiency. Currently, administration of cryoprecipitated antihemophilic factor (AHF), which is also called factor VIII, is the preferred treatment for fibrinogen replacement. Each bag of cryoprecipitated AHF contains about 250 mg of fibrinogen. A plasma level of at least 50 mg/dl of fibrinogen is required for adequate hemostasis with surgery or trauma. See, M. S. Kennedy: "Transfusion Therapy". *In* D. Harmening-Pittiglio et al. (eds.), *Modern Blood Banking and Transfusion Practices*, 2nd ed., Philadelphia, F. A. Davis Company, 1989, page 268. Administration of recombinant fibrinogen or variants thereof to such patients will be by the intravenous route and the typical daily dosage will be such as is necessary to

maintain the minimum plasma level of 50 mg/dl of fibrinogen. For such purposes, recombinant fibrinogen or variants thereof can be added to whole blood or blood products, e.g., plasma, cryoprecipitates etc., or to the conventional pharmaceutical vehicles.

The invention will now be described in greater detail by reference to the following non-limiting examples:

Materials: The expression vector (pYES2) and the yeast strain (INSVC1, MATα his3-Δ1 leu2 trp1-289 ura3-52) were obtained from Invitrogen, Inc. Medium to grow the yeast in selective conditions was purchased from Bio101, Inc. Galactose, raffinose, tunicamycin were obtained from Sigma. Antibodies to human fibrinogen was from Dako Corporation, restriction enzymes, Klenow fragment, calf intestinal phosphatase (CIP) were from Boehringer, Mannheim, endoglycosidase-H from Genzyme, T4 DNA ligase from New England Biolab, L-[^{35}S] methionine (1100 Ci/mmol) was from New England Nuclear Corporation-Du Pont. Other reagents used have been described previously (see S.N. Roy et al., *J. Biol. Chem., 267:* 23151 (1992); S.N. Roy et al., *J. Biol. Chem., 269:* 691 (1994); and S.N. Roy et al., *J. Biol. Chem., 266:* 4758 (1991)).

Example 1: Construction of Expression Vector

Expression vectors containing fibrinogen cDNAs for single chains, 2 in combinations and all 3 chains are inserted to the multiple cloning sites at the 3' end of the Gal-1 promoter fused with the MFα1 prepro secretion signal (SS) cascade in pYES2 plasmid, which is depicted in Figure 1. To prepare pYES2Aα, pYES2Bß and pYES2γ, full-length cDNAs were released by appropriate restriction enzymes separately from previously described constructs (see S.N. Roy et al., *J.*

Biol. Chem., 269: 691 (1994); and S.N. Roy et al., *J. Biol. Chem., 266:* 4758 (1991)). Other constructs, pYES2AαBß, pYES2Aαγ, pYES2Bßγ and pYES2AαBßγ were made by ligating fibrinogen chain cDNAs in tandem, each under the control of the Gal-1-SS promoter. The procedures for elution of DNA fragments from agarose gel, dephosphorylation of plasmids by CIP, the fill-in reaction by Klenow fragment and ligation were performed as described elsewhere (see S.N. Roy et al., *J. Biol. Chem., 267:* 23151 (1992); S.N. Roy et al., *J. Biol. Chem., 269:* 691 (1994); and S.N. Roy et al., *J. Biol. Chem., 266:* 4758 (1991)).

Example 2: Transformation of Yeast

Transformation of *S. cerevisiae* (INVSC1) with pYES2 vectors containing fibrinogen cDNAs were performed by the alkali-cation method and the cells were plated on SC-ura plates (see L.D. Schultz et al., *Gene, 54:* 113 (1987)). Single colonies from each plate were grown in SC-ura medium containing 4% raffinose at 30°C with vigorous shaking overnight and kept as stock culture. Transformed yeast cells with the above described constructs were named INVSC1Aα, INVSC1Bß and INVSC1γ, INVSC1AαBß, INVSC1Aαγ, INVSC1Bßγ and INVSC1AαBßγ.

S. cerevisiae cells (INVSC1) stably transformed with vector pYES2AαBßγ were prepared in the foregoing manner and deposited with the American Type Culture Collection, Rockville, MD, on August 12, 1994, under accession number ATCC 74296. The deposit was made pursuant to the Budapest Treaty.

Example 3: Expression and Treatment with Tunicamycin

Stock culture was grown in 5 ml of SC-ura medium overnight at a density

of $1x10^8$/ml. The cells were harvested at 500 xg, resuspended in SC-ura medium containing 2% galactose and grown for an additional 16 hr for induction of fibrinogen chain synthesis. The cells were harvested at 500 xg, resuspended in SC-ura-met medium containing 50 μCi/ml of L-[^{35}S]methionine and incubated for 1 hr at 30°C. In some cases, the cells were preincubated with medium containing 10 μg/ml of tunicamycin for 1 hr and L-[^{35}S]methionine as usual. When determining intracellular fibrinogen, the cells were harvested, washed with phosphate buffered saline (PBS), lysed with 0.5 ml of IP buffer (50 mM Tris.HCl, pH 7.4, 1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, 10 U/ml Trasylol, 1 mM PMSF, 0.1 mM TPCK, 1 μg/ml Soyabean-trypsin inhibitor) and 200 mg (0.5 mm dia) of acid-washed glass beads/10^8 cells by vortexing twice for 45 sec (see J.R. Franzusoff et al., *Methods in Enzymology, 194:* 662 (1991)). The cell lysate was diluted to 1 ml with water and centrifuged at 15000 xg for 15 min at 4°C. Fibrinogen was isolated by immunoprecipitating the cytosol with a human polyclonal fibrinogen antibody as described elsewhere (see S.N. Roy et al., *J. Biol. Chem., 267:* 23151 (1992); S.N. Roy et al., *J. Biol. Chem., 269:* 691 (1994); and S.N. Roy et al., *J. Biol. Chem., 266:* 4758 (1991)).

Example 4: Secretion of Fibrinogen

Yeast cells transformed with pYES2AαBßγ and grown from single colony were inoculated in 50 ml of SC-ura medium containing 4% raffinose and grown overnight at 30°C. The cells were then induced with 2% galactose and incubated for an additional 16 hr. The culture medium was centrifuged at room temperature for 5 min at 500 xg. The pH of the medium was adjusted to 7.0 with 1 M Tris-HCl buffer pH 8.0 and a cocktail of protease inhibitors (10 U/ml Trasylol, 1 mM PMSF, 0.1 mM TPCK, 1 μg/ml Soyabean-trypsin inhibitor, 1 mg/ml papstatin) was added. Fibrinogen was isolated from the medium by absorption

on a protamine sulfate-Sepharose 6B column (10 ml) calibrated with buffer A (50 mM Tris.HCl, pH 7.4, 5 mM EDTA). The column was washed with buffer A containing 0.8 M NaCl and bound fibrinogen was eluted with 0.1 M Na-acetate, pH 4.5. The pH was adjusted to 7.0 with 1 M Tris.HCl, pH 8.0 (see C.E. Dempfle et al., *Thromb. Res., 46:* 19 (1987)).

Example 5: Quantitation of Secreted Fibrinogen

The amount of fibrinogen secreted in the medium was measured by competition ELISA using two different fibrinogen chain-specific monoclonal antibodies [1-8C6(anti Bß 1-21) and Fd4-7B3 (anti fibrinogen fragment D)]. Details of the assay using Fd4-7B3 were reported previously (see S.N. Roy et al., *J. Biol. Chem., 266:* 4758 (1991)). A new assay has recently been developed with antibody 1-8C6 whose specificity has been described (see B. Kudryk et al., *Molec. Immun., 20:* 1191 (1983)). A horseradish peroxidase-labeled form of antibody 1-8C6 is utilized in this assay and fibrinogen concentrations as low as 0.05 µg/ml can readily be measured.

Example 6: Comparison of the Properties of Human Plasma and Yeast Recombinant Fibrinogen

Secreted recombinant fibrinogen was treated with thrombin (6.8 NIH U/ml) with or without factor XIII (1.0 U/ml) to determine its ability to form a thrombin-induced clot and to crosslink. The fibrin complexes were separated by SDS-PAGE and detected by staining with coomassie blue and by Western immunoblots using chain-specific antibodies 1C2-2 (anti fibrinogen Aα/fibrin α) (R. Procyk et al., *Thromb. Res., 71:* 127 (1993)); Ea3 (anti fibrinogen Bß/fibrin ß) (B. Kudryk et al., "Monoclonal Antibodies as Probes for Fibrin(ogen) Proteolysis."

In: *Monoclonal Antibodies in Immunoscintigraphy* (J.F. Chatal, ed.), CRC Press, Boca Raton, FL, pp. 365-398); T2G1 (anti fibrin ß) (B. Kudryk et al., *Molec. Immun., 21:* 89 (1984)); and 4-2 (anti fibrinogen γ/fibrin γ-dimer) (R. Procyk et al., *Blood, 77:* 1469 (1991)).

In order to assess the similarity of yeast recombinant fibrinogen to human plasma fibrinogen, comparisons of the two products were made on polyacrylamide gels.

Figure 2 shows the immunoreactivity of recombinant fibrinogen with chain specific antibodies. Recombinant fibrinogen was separated on 4-10% gradient SDS-PAGE under reducing or non-reducing conditions and analyzed by Western immunoblots using different chain specific antibodies.

Panel A: Reduced gel stained with coomassie blue.

Panel B: Immunoblot of reduced samples reacted with MAb to Aα/α chain.

Panel C: Same as B reacted with MAb to Bß/ß chain.

Panel D: Same as B reacted with MAb to γ chain.

Panel E: Immunoblot of non-reduced samples reacted with MAb to γ chain.

Lane 1, molecular size markers; lane 2, plasma Fbg; Lane 3, recombinant yeast Fbg.

The data shows that recombinant fibrinogen secreted by transformed yeast cells has similar electrophoretic and immunoreactive properties as plasma fibrinogen.

Figure 3 shows the clotting properties of recombinant yeast fibrinogen. Purified recombinant fibrinogen secreted by yeast cells was incubated with

thrombin or thrombin + factor XIII at 37°C for 4 hr. After clotting, each sample was solubilized in a DTT and SDS-containing buffer, separated by SDS-PAGE (5-15% gradient gel) and Western blot analysis performed with MAb which reacts with fibrin ß chain or with fibrinogen γ chain or fibrin γ-dimer.

Panel A: Immunoblot stained with coomassie blue.

Panel B: Immunoblot reacted with fibrin ß-chain (T2G1) antibody.

Panel C: Immunoblot reacted with fibrinogen γ chain/fibrin γ-dimer (4-2) antibody.

Lane 1, molecular size markers; Lane 2, plasma Fbg; Lane 3, yeast Fbg; Lane 4, non cross-linked fibrin prepared from yeast Fbg; Lane 5, factor XIIIa cross-linked fibrin prepared from yeast Fbg.

The data shows that recombinant fibrinogen, like plasma fibrinogen, is capable of forming a thrombin-induced clot and undergoing factor XIII induced cross-linking.

It will be appreciated that the instant specification and claims are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED IS:

1. An expression vector for yeast comprising at least one cDNA encoding a polypeptide chain of fibrinogen or a variant thereof.

2. An expression vector according to claim 1, comprising a cDNA encoding the Aα chain of fibrinogen or a variant thereof, a cDNA encoding the Bß chain of fibrinogen or a variant thereof, and a cDNA encoding the γ chain of fibrinogen or a variant thereof.

3. An expression vector according to claim 2, which is a pYES2 expression vector, into which the three cDNAs have been inserted in tandem at the 3′ end of a T7 promoter.

4. An expression vector according to claim 3, wherein the three cDNAs are under the control of the Gal-1-promoter.

5. Yeast transformed with an expression vector according to claim 1.

6. Yeast transformed with an expression vector according to claim 2.

7. Yeast transformed with an expression vector according to claim 3.

8. Yeast transformed with an expression vector according to claim 4.

9. A method of expressing fibrinogen or a variant or subunit thereof in yeast, comprising:

(a) constructing or obtaining an expression vector for yeast containing at least one cDNA encoding a polypeptide chain or fibrinogen or a variant thereof;

(b) transforming yeast with said expression vector and selecting for stable transformants;

(c) maintaining the transformants in a culture medium under conditions wherein fibrinogen or a variant or subunit thereof is secreted into said culture medium; and

(d) recovering fibrinogen or the variant or subunit thereof from the culture medium.

10. A process according to claim 9, wherein said expression vector comprises a cDNA encoding the Aα chain of fibrinogen or a variant thereof, a cDNA encoding the Bß chain of fibrinogen or a variant thereof, and a cDNA encoding the γ chain of fibrinogen or a variant thereof.

11. A process according to claim 10, wherein said expression vector is a pYES2 expression vector, into which the three cDNAs have been inserted in tandem at the 3' end of a T7 promoter.

12. A process according to claim 11, wherein the three cDNAs are under the control of the Gal-1-promoter.

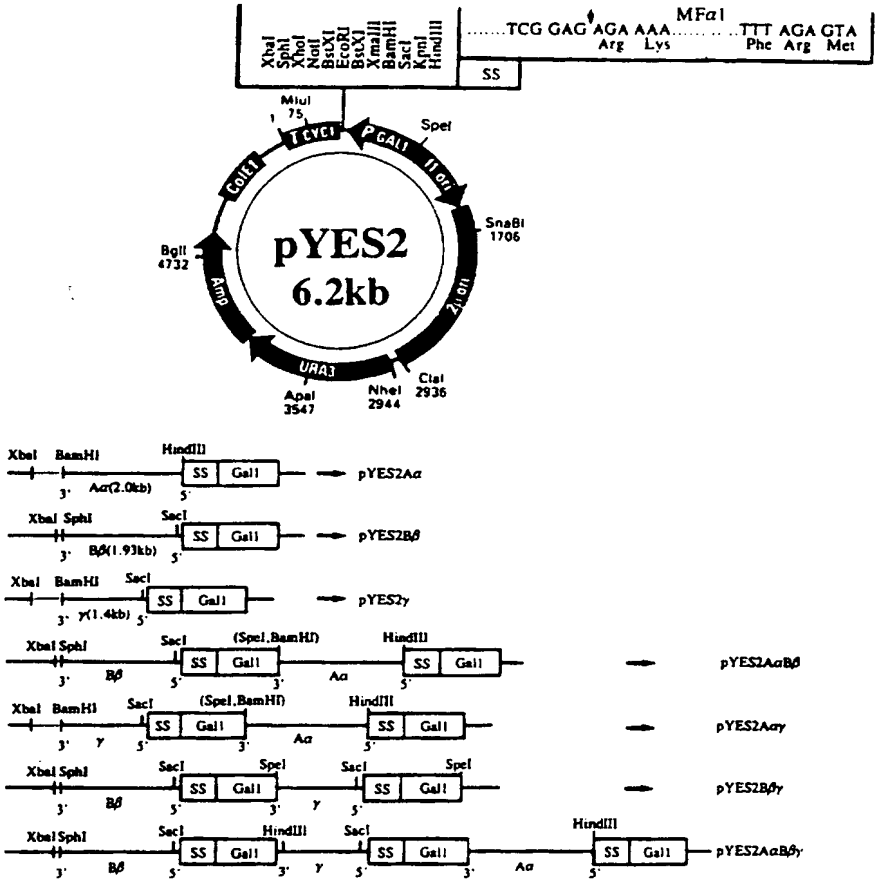
13. Fibrinogen or variant thereof produced according to the process of

claim 9.

14. Fibrinogen or variant thereof produced according to the process of claim 10.

15. Fibrinogen or variant thereof produced according to the process of claim 11.

16. Fibrinogen or variant thereof produced according to the process of claim 12.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MFα1
SS
MluI 75
T CYC1
P GAL1
SpeI
11 ori
SnaBI 1706
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ColE1
pYES2
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FIG. 1

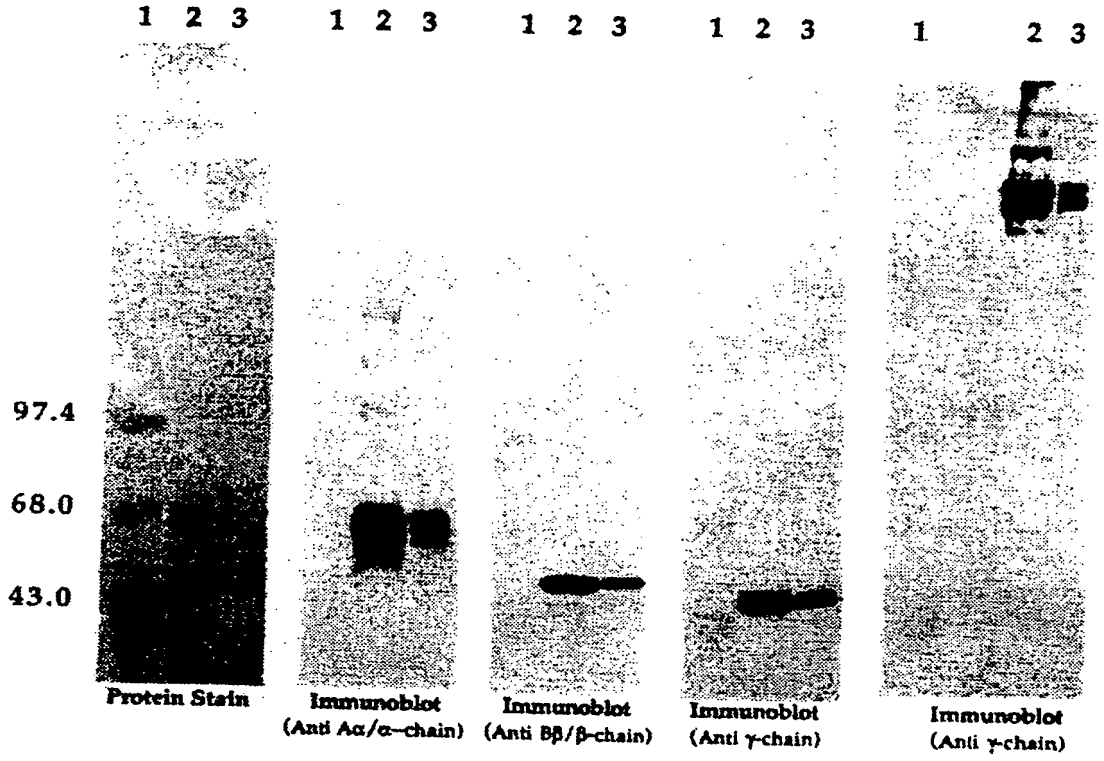

FIG. 2

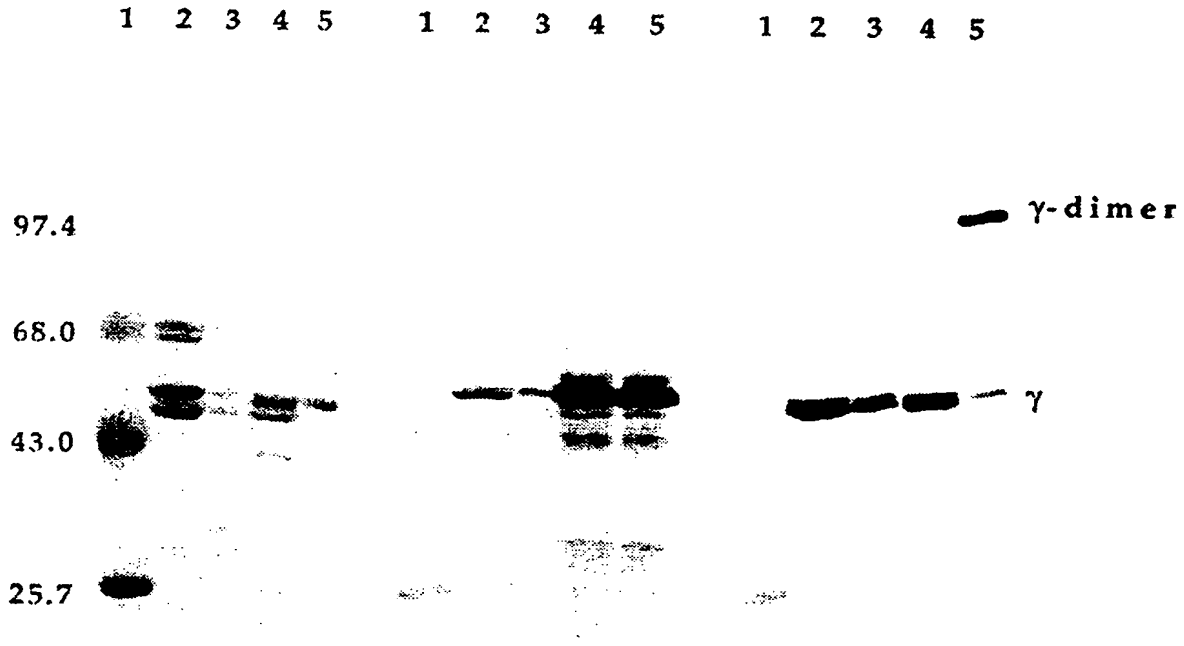
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
97.4
68.0
43.0
25.7
γ-dimer
γ
Protein Stain
Immunoblot
(Anti β-chain)
Immunoblot
(Anti γ-chain)

FIG.3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11139

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 254.11, 254.2, 320.1; 530/350; 536/22.1, 23.1, 23.4, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BIOCHEMISTRY, Vol. 22, No. 13, issued 1983, Chung et al., "Characterization of a Complementary Deoxyribonucleic Acid Coding for the gamma Chain of Human Fibrinogen", pp. 3250-3256, see entire document.	13-16 ------- 1-12
X --- Y	BIOCHEMISTRY, Vol. 22, No. 13, issued 1983, Chung et al., "Characterization of Complementary Deoxyribonucleic Acid and Genomic Deoxyribonucleic Acid for the beta Chain of Human Fibrinogen," pp. 3244-3250, see entire document.	13-16 ------- 1-12
X --- Y	BIOCHEMISTRY, Vol. 22, No. 13, issued 1983, Rixon et al., "Characterization of a Complementary Deoxyribonucleic Acid Coding for the alpha Chain of Human Fibrinogen," pp. 3237-3244, see entire document.	13-16 ------ 1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 DECEMBER 1995	Date of mailing of the international search report 28 DEC 1995
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11139

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH, Vol. 20, No. 4, issued 1992, Marcil et al ., "Direct transfer of plasmid DNA from yeast to E. coli by electroporation," p. 917, see entire document.	1-16
Y	GENE, Vol. 118, issued 1992, Rokeach et al., "Overproduction of a human snRNP-associated Sm-D autoantigen in Escherichia coli and Saccharomyces cerevisiae," pp. 247-253, see entire document.	1-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11139

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 1/14, 1/16, 1/18, 15/00; C12P 21/06; C07H 19/00, 21/00; C07K 1/00, 2/00, 4/00, 14/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 254.11, 254.2, 320.1; 530/350; 536/22.1, 23.1, 23.4, 23.5

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, BIOSIS
search terms: fibrinogen, yeast, recombinant production of fibrinogen in yeast, pyes2, invsc?, recombinant, galactokinase, gal1, gal-1, promoter, t7